

# Identification of Male-Specific AFLP Markers in Dioecious Texas Bluegrass

K. Renganayaki, R. W. Jessup, B. L. Burson,\* M. A. Hussey, and J. C. Read

## ABSTRACT

Dioecy is a breeding system that promotes cross-pollination in plants. The transfer of this trait into economically important self-pollinated cereal crops would revolutionize the production of hybrids in these species and provide a means for increasing yields because of heterosis. Texas bluegrass (*Poa arachnifera* Torr.) ( $2n = 8x = 56$ ) is a polymorphic dioecious species that provides an opportunity to genetically map the dioecy locus. In this study, amplified fragment length polymorphism (AFLP)-based linkage maps were constructed for both the maternal and paternal plants used to develop a mapping population of Texas bluegrass. The maternal map contained 126 single dose restriction fragments (SDRFs), 31 linkage groups, 1744 cM, and an average marker spacing of 13.8 cM. The paternal map contained 210 SDRFs, 46 linkage groups, 2699 cM, and an average marker spacing of 12.9 cM. Approximately 76 to 81% of the Texas bluegrass genome was covered. Two AFLP markers (*txbg7* and *txbg154*) mapped equidistantly (9.5 cM) on opposite sides of the dioecy locus (*PDio1*) on the paternal map. These markers provide a preliminary tool for studying sex determination and a framework for further characterization of the genomic region conferring dioecy in Texas bluegrass.

APPROXIMATELY 90% of the flowering plants in the world are hermaphroditic in that the female and male reproductive organs are in the same flower (Ainsworth, 2000; Charlesworth, 2002). However, in some species, hermaphroditic flowers have evolved several mechanisms that increase cross-pollination, which avoids inbreeding depression and promotes heterozygosity, genetic variability, and genetic exchange (Dellaporta and Calderon-Urrea, 1993). These mechanisms include protogyny, chasmogamy, heterostyly, self-incompatibility, self-sterility, entomophily, anemophily, and hydrophily. The remaining flowering plants have evolved physically separated, unisexual flowers. Approximately half of these are monoecious, and the male and female reproductive organs develop in separate flowers on the same plant. Monoecy prevents intraflower self-pollination but does not necessarily prevent intraindividual self-pollination (Charlesworth, 2002). The remaining plants are dioecious in that individual plants within the same species have either male or female flowers. Dioecy prevents intraindividual self-pollination and is one of the most extreme inbreeding avoidance mechanisms (Ainsworth, 2000). Dioecy occurs throughout the angiosperms; however, it

is more common among the dicots than the monocots (Renner and Ricklefs, 1995).

*Poa* is a large, diverse genus that belongs to the subfamily Pooideae of the Poaceae. Several species are complex polyploids with a range of breeding systems, including apomixis and dioecy, that promote interspecific hybridization and introgression (Muntzing, 1940; Clausen, 1961). Because of this, it is frequently difficult to taxonomically distinguish one species from another. Texas bluegrass ( $2n = 8x = 56$ ) is a dioecious, perennial cool-season grass that is native to southern Kansas, Oklahoma, western Arkansas, and most of Texas (Hitchcock, 1950; Gould, 1975). It is drought and heat tolerant and produces high quality forage in regions where other cool-season temperate grasses, such as tall fescue (*Festuca arundinacea* Schreb.), are not sustainable (Read et al., 1997). The grass grows throughout the winter but is usually dormant during the summer (Magness et al., 1971). However, it produces only limited quantities of seed that are covered with woolly hairs that are difficult to remove. Consequently, establishment of stands for agricultural use is difficult. The species has been successfully crossed with Kentucky bluegrass (*Poa pratensis* L.) in an effort to combine heat and drought tolerance of Texas bluegrass with high forage and turf quality of Kentucky bluegrass (Vinall and Hein, 1937; Read et al., 1999; Bonos et al., 2000; Abraham et al., 2004). 'Reveille', the first Texas bluegrass × Kentucky bluegrass hybrid cultivar released, is a heat tolerant turfgrass adapted to the southwestern USA (Read et al., 1999). Hybrids between dioecious Texas bluegrass and apomictic Kentucky bluegrass are facultative apomicts and demonstrate the reproductive versatility within *Poa*.

Although little molecular information is available regarding Texas bluegrass, a study using AFLP markers investigated the diversity within the species and determined that genetic mapping is feasible (Renganayaki et al., 2001). Dioecy is a trait that is of considerable interest in mapping, and molecular markers have been linked to dioecy in a number of angiosperms (Ainsworth, 2000; Charlesworth, 2002; Vyskot and Hobza, 2004). However, most of these are dicots and little has been done in the monocots, especially members of the Poaceae family. According to Watson and Dallwitz (1992 onward), there are 22 grass genera that have at least one dioecious species, and of these, a molecular study addressing sex determination has been conducted for only one species. Eppley et al. (1998) reported a single random amplified polymorphic DNA (RAPD) marker linked to the dioecy trait in salt grass [*Distichlis spicata*

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**Abbreviations:** AFLP, amplified fragment length polymorphism; cM, centimorgan; PCR, polymerase chain reaction; RAPD, random amplified polymorphic DNA; SDRF, single dose restriction fragment; TBF, Texas blue forage; TIFF, tagged image file format.

(L.) Greene]; however, this class of marker has limited utility in comparative studies across other dioecious grasses. In addition, dioecy appears to have evolved independently on numerous occasions (Ainsworth, 2000; Charlesworth, 2002). As a result, markers associated with the trait in a given species would have limited utility in comparative studies of different species.

The molecular characterization of dioecy in Texas bluegrass could provide valuable tools for plant breeders working with cereal, forage, and turf grasses. The identification and transfer of genes controlling dioecy across grass species could simplify hybrid production in self-pollinated crops [i.e., rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), and sorghum [*Sorghum bicolor* (L.) Moench]]. A genome map of Texas bluegrass would also attenuate the sparsity of molecular information available for marker-assisted breeding efforts in perennial forage and turf grasses. Accordingly, the objectives of this study were to (i) construct a genetic linkage map of the Texas bluegrass genome and (ii) identify molecular markers linked to the dioecy locus.

## MATERIALS AND METHODS

### Plant Materials

A female plant [Texas blue forage, TBF 20–16 (3–88)] and a male plant [TBF 10–25 (3–88)] were selected from the same Texas bluegrass ecotype and used to initiate a genetic mapping study of dioecy. TBF 20–16 was crossed with TBF 10–25 and 100 diverse  $F_1$  hybrids were selected and used as the mapping population.

### DNA Isolation

Young leaves were collected from both parents and all 100 of the  $F_1$  hybrids in the mapping population, frozen at  $-80^\circ\text{C}$ , and lyophilized for 48 h. Genomic DNA was extracted from the lyophilized leaf tissue as described by Williams and Ronald (1994). The genomic DNA was quantified with a DYNA Quant 200 fluorometer (Hoefer Pharmacia Biotech, San Francisco, CA) using the manufacturer's protocols.

### Analysis of AFLP Markers

AFLPs were generated using the protocol of Vos et al. (1995) with minor modifications. Genomic DNA of the parents and the mapping population was completely and sequentially digested with the restriction enzymes *MseI* and *PstI* (New England Biolabs, Beverly, MA). Restricted genomic DNA fragments were ligated to *MseI* and *PstI* adapters overnight at  $37^\circ\text{C}$ . Restricted and ligated DNA was diluted to a final concentration of  $1\text{ ng}/\mu\text{L}$ . Preamplification of the diluted template DNA was performed with AFLP primers that have one selective nucleotide at the 3' end (*PstI* + 1 and *MseI* + 1) as described by Vos et al. (1995). Twenty-microliter polymerase chain reaction (PCR) reactions were performed containing 5- $\mu\text{L}$  template DNA, 14 pmole each of *PstI* + 1 and *MseI* + 1 primers, 1 U *Taq* DNA polymerase (Gibco BRL, Carlsbad, CA),  $1\times$  PCR buffer, 1.5 mM  $\text{MgCl}_2$ , and 200  $\mu\text{M}$  dNTPs. Preamplification reactions were performed for 25 cycles: 30 s at  $94^\circ\text{C}$ , 1 min at  $56^\circ\text{C}$ , and 1 min at  $72^\circ\text{C}$ . Preamplification products were diluted (1:10) with  $\text{ddH}_2\text{O}$  and used as templates for selective amplification. Selective amplification was performed with primers with three selective nucleotides. Infra Red Dye (IRD)-labeled *PstI* primers, obtained from LI-COR

Inc. (Lincoln, NE), were diluted according to manufacturer's recommendations. Selective amplification reactions were performed in a final volume of 10  $\mu\text{L}$  containing 2  $\mu\text{L}$  of diluted preamplification DNA (50 pg), 2.5 pmole of *MseI* selective primer, 0.25  $\mu\text{L}$  of IRD labeled *PstI* selective primer, 0.2 U *Taq* DNA polymerase (Promega Corp., Madison, WI),  $1\times$  PCR buffer, 2.5 mM  $\text{MgCl}_2$ , and 200  $\mu\text{M}$  dNTPs. Selective amplification was performed for 2 min at  $95^\circ\text{C}$  followed by 13 cycles of 1 min at  $94^\circ\text{C}$ , 1 min at  $65^\circ\text{C}$  and 1.5 min at  $72^\circ\text{C}$  with a  $0.6^\circ\text{C}$  reduction in annealing temperature in each cycle. This was followed by 22 cycles of 30 s at  $94^\circ\text{C}$ , 30 s at  $56^\circ\text{C}$  and 1 min at  $72^\circ\text{C}$  with a final extension at  $72^\circ\text{C}$  for 5 min. Selectively amplified PCR products were analyzed with a LICOR model 4200 dual dye automated DNA sequencing system. An equal volume (5  $\mu\text{L}$ ) of IRD 700 and IRD 800 nm labeled PCR products was pooled, along with 2  $\mu\text{L}$  of basic fuchsin dye. This was denatured for 5 min at  $95^\circ\text{C}$ . One microliter of the denatured sample was loaded on a 6.5% (w/v) polyacrylamide gel containing 7 M Urea. Electrophoresis was performed for 3 to 3.5 h at a constant power of 40 W and a constant temperature of  $47.5^\circ\text{C}$ .

### Linkage Analysis

TIFF (Tagged Image File Format) images obtained from a LI-COR DNA sequencer were analyzed by RFLP Scan plus 3.12 software (Scanalytics, Inc., Fairfax, VA). Bands present in one parent and absent in the other parent were scored for presence and absence ( $\pm$ ) in the progeny and were numbered serially with a prefix "txbg". Polymorphic markers were tested for a fit to a 1:1 Mendelian segregation by  $\chi^2$  analysis at 5% significant level to identify the SDRFs. Because SDRFs only reveal recombination in the gametes of one parent, an AFLP linkage map was constructed for each parent by Mapmaker version 3.0 (Lander et al., 1987). All pairs of linked markers were identified by the "Group" command with a LOD score of 3.0 and recombination fraction of 0.50. The ripple command was used to verify the order of markers in each linkage group. Map units in centimorgans (cM) were derived from the Kosambi function (Kosambi, 1944), and the map was drawn by MapChart 2.1 (Voorrips, 2002).

### Estimation of Genome Size and Coverage

Genome length,  $G$ , was estimated from partial linkage data using the equation  $G = MX/K$  (Hulbert et al., 1988), where  $M$  = number of informative meioses,  $X$  = an interval in centimorgans at some minimum LOD score, and  $K$  = actual number of pairs of markers observed that border the interval  $X$  or less. The proportion of genome coverage  $C$ , in terms of probability ( $P$ ) of a random point not being covered, was calculated by the following equation as suggested by Bishop et al. (1983):  $C = 1 - (2r)/(n+1)[(1 - X/2t)^{n+1} - (1 - X/t)^{n+1}] + (1 - rX/t)(1 - X/t)^n$ , where  $r$  = number of linkage groups,  $X$  = an interval in centimorgans,  $n$  = the number of intervals, and  $t$  = the sum of linkage group lengths in centimorgans.

### Chromosome Pairing Analysis

To detect repulsion-phase linkage, two-point linkage analyses were repeated with inverted marker scores (Al-Janabi et al., 1993; Jessup et al., 2003). A 1:1 ratio of markers in repulsion- vs. coupling-phase within each linkage group would indicate allopolyploidy (disomic inheritance), and a 0.25:1 ratio would indicate autopolyploidy (tetrasomic inheritance). Intermediate ratios would suggest partial preferential or multivalent chromosome pairing and a more complex form of polyploidy (Wu et al., 1992).

## RESULTS

### Segregation of Sex Determination

The F<sub>1</sub> mapping population consisted of 58 female and 42 male plants. Chi-square calculations as used by Wu et al. (1992) revealed that segregation for sex determination in the hybrids did not deviate from the 1:1 segregation ratio ( $\chi^2 = 2.56$ ,  $\chi^2_{1,0.01} = 6.63$ ,  $P = 0.11$ ) expected for a single-dose allele.

### AFLP Segregation Analysis

A total of 140 AFLP primer combinations were tested on both parents and 100 F<sub>1</sub> hybrids. These 140 primer combinations yielded 681 polymorphic markers, with a range of one to 17 and an average of 4.8 markers per primer combination. Of the 681 polymorphic markers, 473 segregated in a 1:1 ratio. The remaining 208 markers significantly deviated from the Mendelian segregation ratio of 1:1 on  $\chi^2$  analysis. Of the 242 and 439 markers unique to the maternal (TBF 20–16) and paternal (TBF 10–25) parents, 184 (72.7%) and 289 (61.0%) markers segregated as SDRFs (Table 1), respectively. A total of 30% of the polymorphic markers had a skewed segregation, and the proportion of markers with a skewed segregation ratio was higher in the paternal parent (34%) than the maternal parent (24%).

### Map Construction

Separate maternal and paternal linkage maps were constructed by means of 184 and 289 SDRFs, respectively. The dioecious phenotypic data was also included for mapping. The maternal linkage map was made up of 31 linkage groups with an average of 4.1 markers per group (Fig. 1). Fifty-eight markers remained unlinked. The maternal map covered a total length of 1744 cM and had an average distance between markers of 13.8 cM. There was substantial linkage group variation in the maternal map. Four large linkage groups spanned from 180 to 291 cM and contained 8 to 14 markers, 5 linkage groups spanned 58 to 93 cM and contained 5 to 9 markers, and the remaining 22 linkage groups spanned 3 to 40 cM and contained 2 to 6 markers. Of the 289 SDRFs segregating in the gametes of the paternal parent, 210 were mapped into 46 linkage groups with an average of 4.6 markers per group. The remaining 79 markers could not be assigned to any linkage group. The paternal map covered 2699 cM and had an average distance of 12.9 cM between markers (Fig. 2). The length and marker content of the linkage groups varied greatly in the paternal map. Eight large linkage groups spanned from 105 to 226 cM and contained more than 7 markers, 14 linkage groups spanned from 52 to 92 cM and contained 3 to 8 markers, and the remaining 24 linkage groups spanned from 8 to 44 cM and contained 2 to 5 markers.

### Genome Size and Coverage

Genome size was estimated by markers spaced at 20 cM or less according to Hulbert et al. (1988). The

**Table 1. Analysis of polymorphic SDRF markers in the Texas bluegrass mapping population.**

	Female parent <sup>†</sup>	Male parent <sup>‡</sup>	Total <sup>§</sup>
Total number of markers analyzed	242	439	681
Markers segregated for 1:1 ratio	184	289	473
Markers deviating from the expected ratio at $P = 0.05$ (distorted segregation)	58 (24%)	150 (34%)	208 (30%)

<sup>†</sup> Polymorphic markers segregating for female parent.

<sup>‡</sup> Polymorphic markers segregating for male parent.

<sup>§</sup> Total number of polymorphic markers observed.

estimated size of the genome based on mapping data from maternal and paternal gametes was 2582 and 4664 cM, respectively. The proportion of the genome coverage was calculated as 76 and 81% for the maternal and paternal parents, respectively.

### Chromosome Pairing

Repulsion analyses identified 6 and 10 maternal and paternal linkage groups, respectively, which fit the 1:1 ratio expected for disomic chromosome pairing. All of the remaining linkage groups fit the 0.25:1 ratio expected for tetrasomic inheritance.

### Identification of Markers Linked to Dioecy

The primer combinations P-CGT+M-CAC and P-CTC+M-CCT produced 112-bp (*txbg7*) and 154-bp (*txbg154*) sized fragments that were present in the paternal parent but absent in the maternal parent. When these two primer combinations were tested on F<sub>1</sub> hybrids, *txbg7* was present in all but four male progeny and *txbg154* was present in all but eight male progeny. No female-specific markers were observed. The two male-specific markers mapped equidistantly (9.5 cM) on opposite sides of the dioecy locus (*PDio1*) on linkage group 41 (Fig. 2). Pairwise marker comparisons using the “BIG LODS” function of MAPMAKER/EXP 3.0 further confirmed close genetic linkage between *PDio1* and *txbg7* (LOD = 13.81,  $r = 0.10$ ) and *txbg154* (LOD = 13.81,  $r = 0.10$ ).

## DISCUSSION

A substantial number of AFLP markers were recovered and are suitable for genetic mapping in Texas bluegrass. The average number of polymorphic markers per primer combination obtained (4.8) was less than that observed in ryegrass (*Lolium perenne* L.) (Bert et al., 1999) but higher than that in *Saccharum spontaneum* L. (Al-Janabi et al., 1993).

Several findings from this study indicate that gaps remain in the Texas bluegrass genome map. On the basis of analyses of linkage map data, we estimate 76 and 81% coverage of the maternal and paternal genomes, respectively. Neither the female nor male map contained the 56 linkage groups expected, on the basis of the full chromosome complement of Texas bluegrass ( $2n = 8x = 56$ ) (Clausen and Hiesey, 1958; Read and Anderson, 2003). The occurrence of small (<40 cM) linkage groups, large map intervals (>20 cM) between

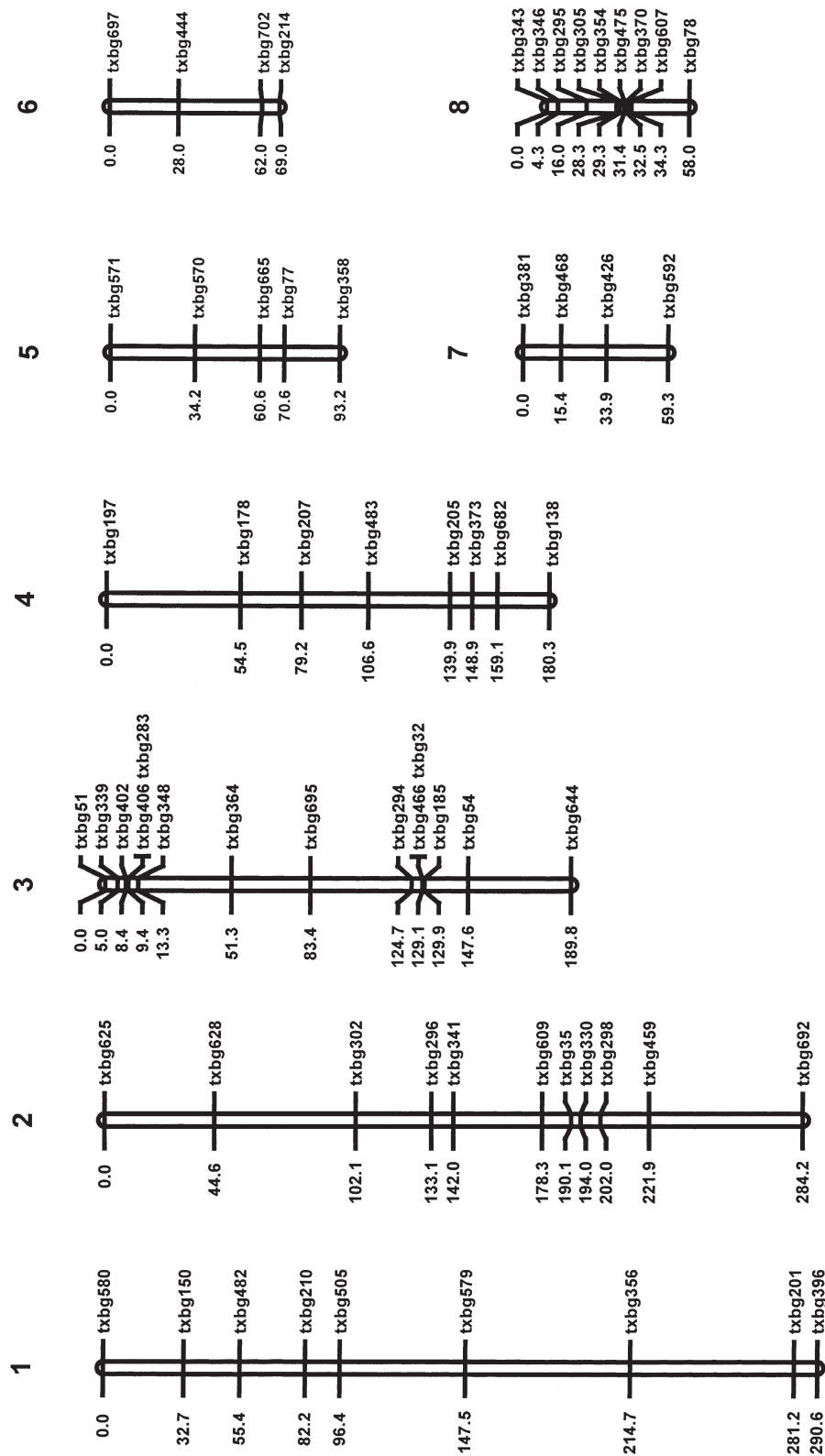


Fig. 1. Continued on next page.

some adjacent markers, and unlinked markers indicate the need for additional marker saturation of the maps. Genome size estimates differed between the parental maps. This finding may be due to variability in marker distribution along the chromosomes, recombination fre-

quencies between the maternal and paternal gametes, or statistical and experimental error (Wu et al., 2004). The Texas bluegrass genome size estimate of 1744 to 2699 cM is lower than that reported in buffelgrass [*Penisetum ciliare* (L.) Link syn. *Cenchrus ciliaris* L.] (2757–

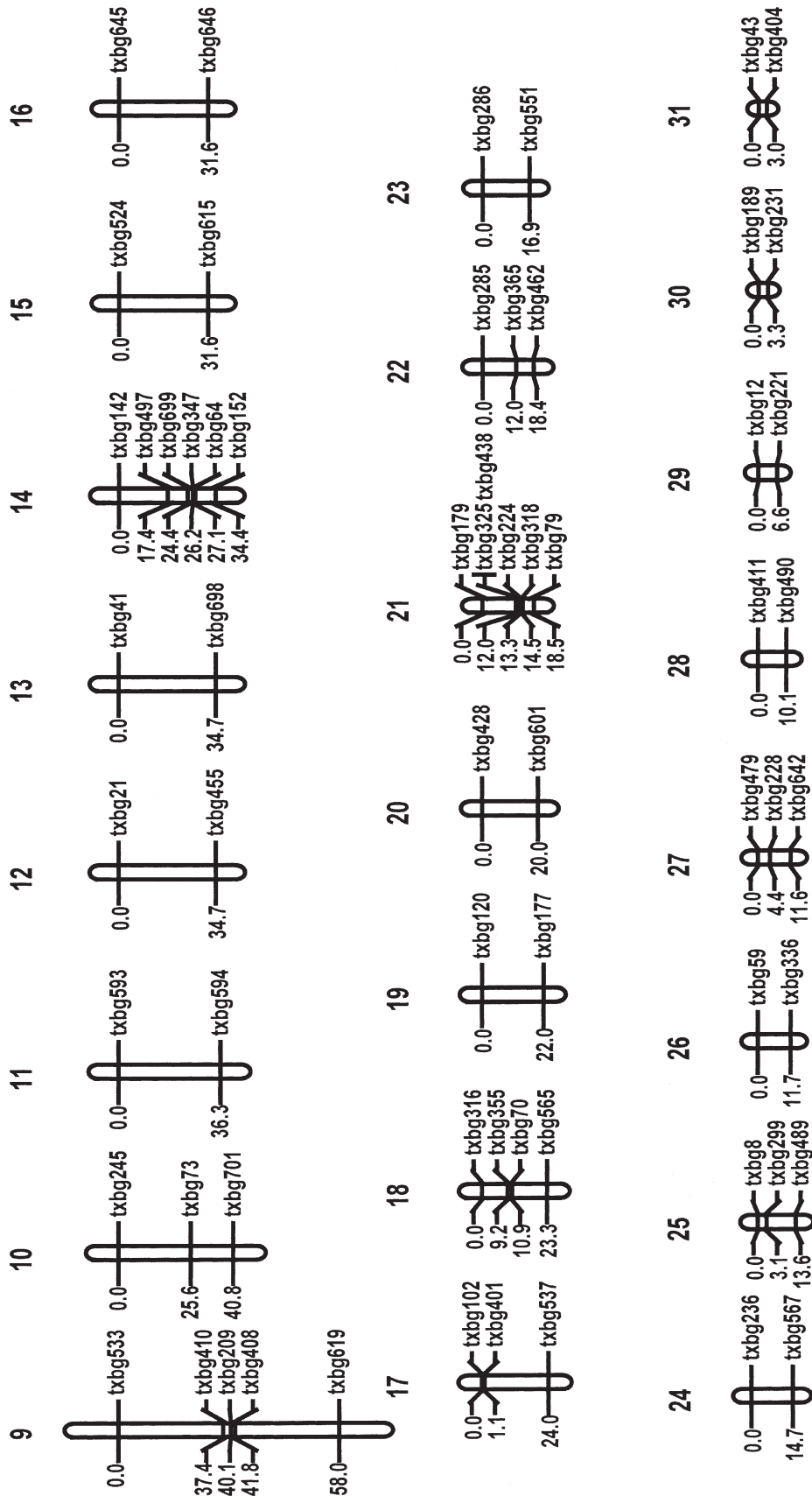


Fig. 1. AFLP genetic linkage map of the maternal parent TBF 20-16 (3-88) with markers on the right side and the distance in centimorgans on the left.

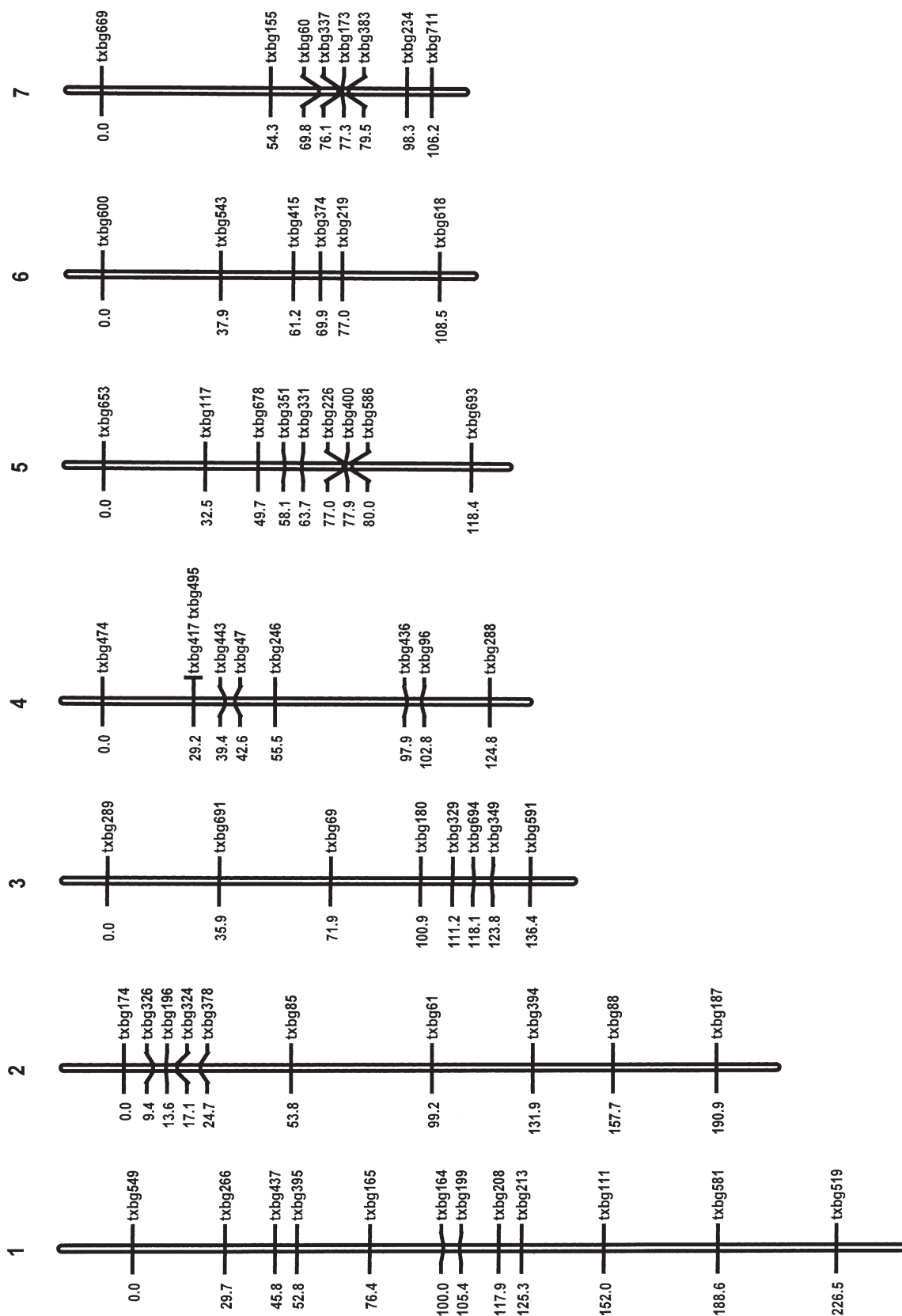


Fig. 2. Continued on next page.

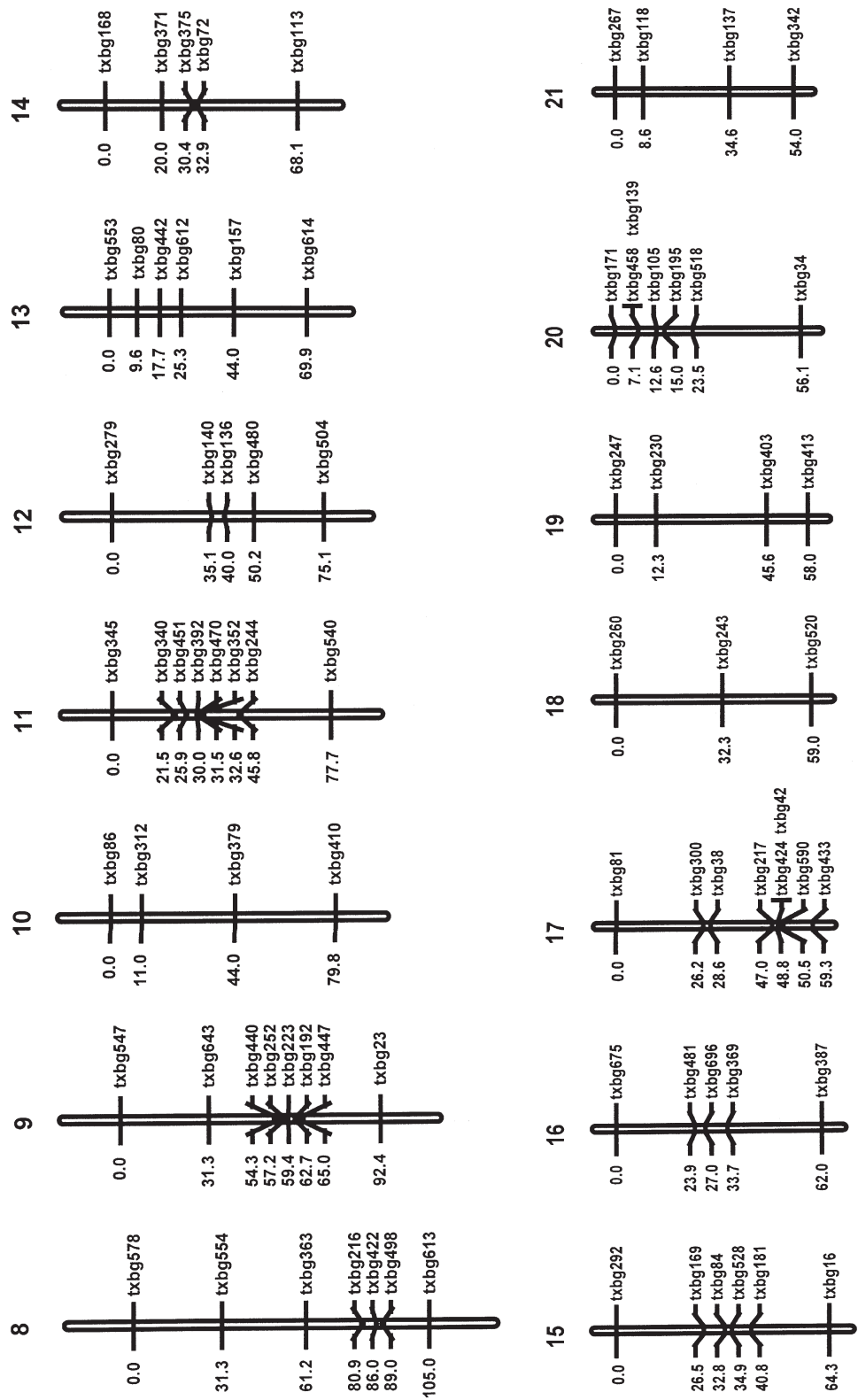


Fig. 2. Continued on next page.

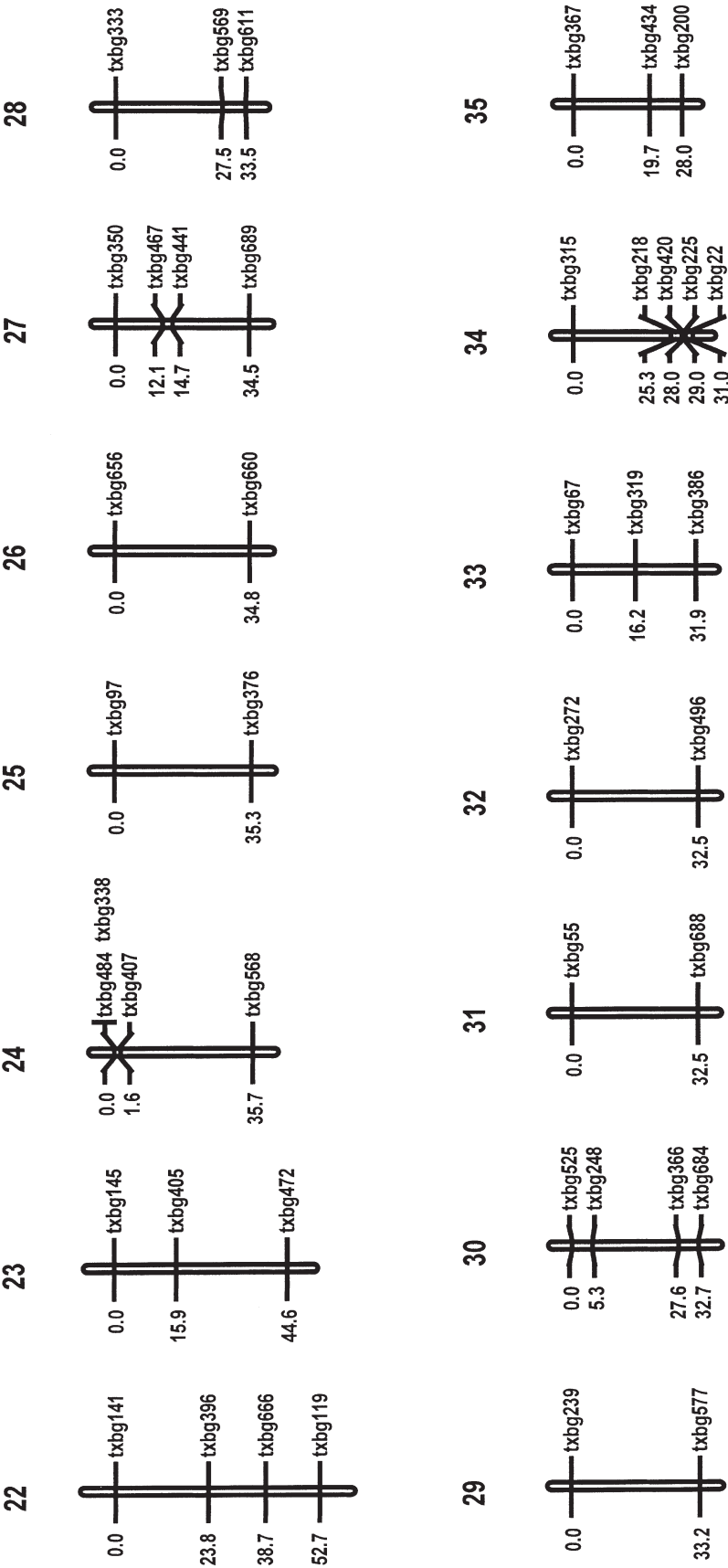


Fig. 2. Continued on next page.

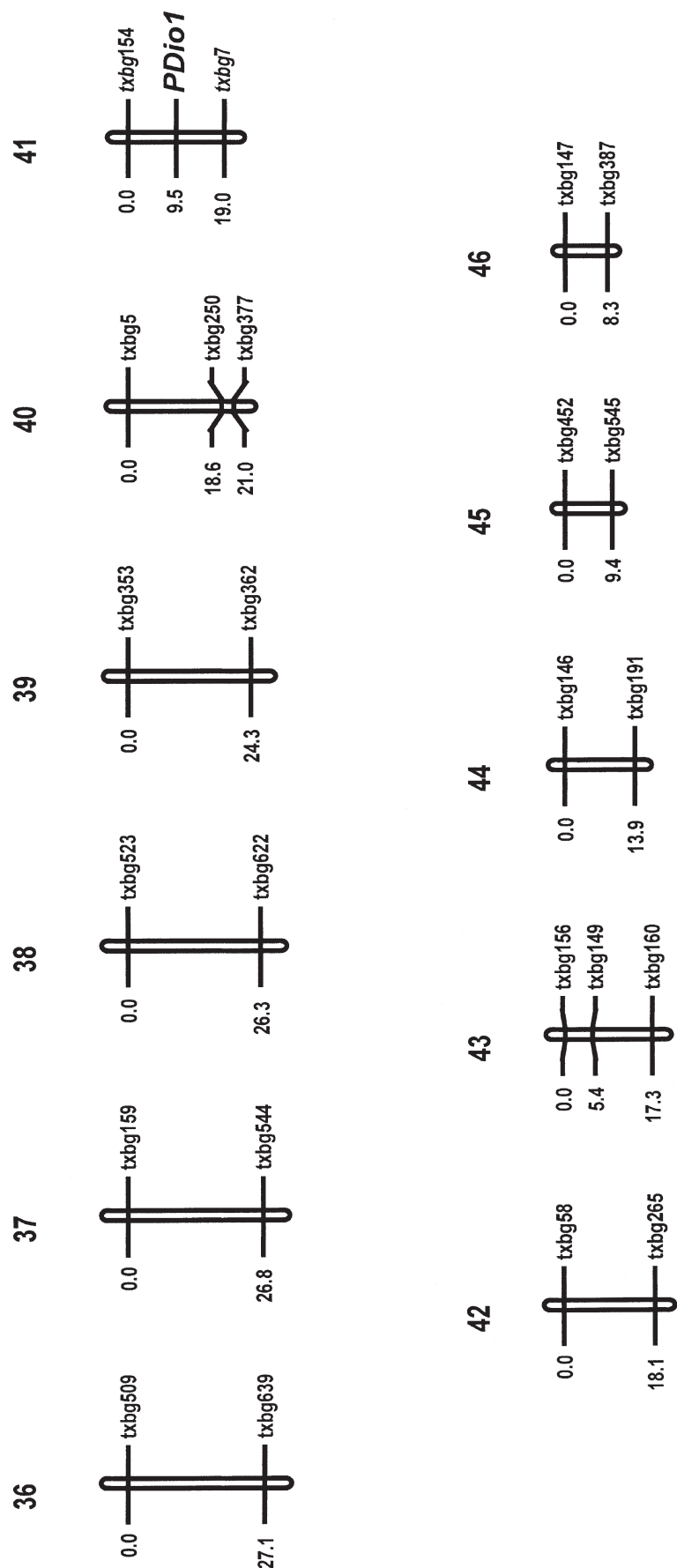


Fig. 2. AFLP genetic linkage map of the paternal parent TBF 10-25 (3-88) with markers on the right side and the distance in centimorgans on the left.

3464 cM) (Jessup et al., 2003) but higher than that reported in *S. spontaneum* (2550 cM) (Al-Janabi et al., 1993).

Because Texas bluegrass is an octaploid with an unknown meiotic chromosome pairing behavior, several genetic segregation ratios are possible. However, the fact that a large fraction (30%) of markers in this study did not have monogenic (1:1) segregation indicates that segregation distortion may have occurred in the mapping population. Segregation distortion has been recognized through linkage mapping studies in many plants since the advent of molecular markers. Segregation distortion can vary from 0% (Beavies and Grant, 1991) to 80% (Paterson et al., 1991) and is more prevalent in mapping populations derived from interspecific hybridization (Wu et al., 2003) or inbreeding (Osborn et al., 1998). A portion of this deviation from a 1:1 segregation ratio could be due to the low level of segmental polyploidy identified in the Texas bluegrass genome through repulsion analyses. In addition, the segregation distortion could be caused by (i) chromosome pairing factors, (ii) favored allelic responses, (iii) natural selection toward one parent, (iv) association between heterozygosity and plant vigor, (v) genes linked to incompatibility loci, (vi) superimposition of non-allelic amplified products corresponding to different loci on gels, and (vii) size differences between parental genomes.

Even though the meiotic chromosome pairing behavior of this complex octoploid species is not established, some inferences regarding the chromosomes and their meiotic behavior can be made from this study. Repulsion analysis results indicate that preferential pairing occurs in a minority of the Texas bluegrass chromosomes but random pairing occurs in a majority of the chromosomes. This chromosome pairing behavior rules out amphiploidy in Texas bluegrass, but it cannot distinguish between the possibility of autooctaploidy, autoallooctaploidy, or an intermediate form of segmental allooctaploidy. In spite of this, the Texas bluegrass genome map can be utilized in future studies to determine what type of chromosome pairing occurs near traits of interest. Such genomic information is important to determine the feasibility of gene isolation and choice of appropriate plant breeding methodologies.

The identification of markers linked to *PDio1* is a first step toward utilizing the Texas bluegrass genome map for marker-assisted plant breeding. Although 9.5 cM occur between each of the male-specific markers and the *PDio1* locus, the simultaneous use of these two flanking markers has a selection efficiency greater than 95%. The discovery of these two male-specific markers but no female-specific markers is similar to what was found in hemp (*Cannabis sativa* L.) (Mandolino et al., 1999) and Garrett's saltbush (*Atriplex garrettii* Rydb.) (Ruas et al., 1998). Recombination between the male-specific markers and the sex-determining locus indicates that this region is not located on a heteromorphic sex chromosome because recombination would be largely absent. This indicates that dioecy is of relatively recent origin in Texas bluegrass and sex chromosomes have not evolved in the species. The finding that *PDio1* is the

only locus exerting control of male sex determination in Texas bluegrass agrees with findings in other plant species and suggests that dioecy is a trait suitable for map-based cloning studies. Unlike other dioecious species with polygamodioecious (intermediate) plants, the strict dioecious nature of Texas bluegrass would facilitate such efforts.

This genome map will be a useful tool for Texas bluegrass improvement. Placing traits of interest onto the map will allow the development of markers useful for marker-assisted selection in Texas bluegrass and other perennial forage and turf grasses. Markers for drought and heat tolerance could potentially be utilized for the introgression of these traits into Texas bluegrass x Kentucky bluegrass hybrids. The two male-specific markers (*txbg7* and *txbg154*) can be directly used to facilitate early sex determination of Texas bluegrass plants. The identification of markers more tightly linked to the *PDio1* locus will facilitate the characterization and isolation of gene(s) controlling this important trait. The eventual transfer of dioecy to other grass species will allow hybrid production in species in which cross-pollination is difficult, as well as simplify hybrid seed production methods currently used in agriculturally important crops.

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